ELSEVIER

Journal of Chromatography A, 812 (1998) 17–34

JOURNAL OF CHROMATOGRAPHY A

Review

Chromatography as an analytical tool for selected antibiotic classes: a reappraisal addressed to pharmacokinetic applications

Antonio Marzo*, Lorenzo Dal Bo

I.P.A.S. S.A., Clinical Pharmacology Department, Via Mastri, Zona Stramonte, 6853 Ligornetto, Switzerland

Abstract

The first antibiotic discovered, penicillin, appeared on the market just after the Second World War. Intensive research in subsequent years led to the discovery and development of cephalosporins, aminoglycosides, tetracyclines and rifamycin. The chemotherapeutic quinolones and the more recently discovered fluoroquinolones have added promising new therapeutic weapons to fight the microbial challenge. The major role pharmacokinetics has played in developing these compounds should be highlighted. Plasma concentration–time profiles and the therapeutic activity evoked by these compounds allow the therapeutic window, doses and dose turnovers to be appropriately defined, as well as possible dose adjustment to be made in renal failure. The pharmacokinetics of antimicrobial agents were initially explored by using microbiological methods, but these lack specificity. The HPLC technique with UV, fluorometric, electrochemical and, in some cases, mass spectrometry detection has satisfactorily solved the problem of antimicrobial agent assay for pharmacokinetic, bioavailability and bioequivalence purposes alike. Indeed, in these studies, plasma concentrations of the given analyte must be followed up for a period $\geq$3 times the half-life, which calls for specific sensitive assays. In this review, the authors have described the analytical methods employed in the pharmacokinetics of antibiotics, including some chemotherapeutic agents which are used in medical practice as alternatives to antibiotics. The pharmacokinetic characteristics of each class of drugs are also briefly described, and some historical and chemical notes on the various classes are given.

Keywords: Reviews; Pharmacokinetics; Kinetic studies; Antibiotics; Lactams; Aminoglycosides; Macrolides; Tetracyclines; Sulfonamides; Quinolones

Contents

*Corresponding author.

0021-9673/98/$19.00
PII: S0021-9673(98)00282-9

1. Introduction

The term antibiosis defines an association between two organisms that is detrimental to one of them, or between one organism and an antibiotic produced by another. The concept and practice of antibiosis originates in ancient Chinese medicine. In 1877, the clinical potential of microbial products as therapeutic agents was recognized by Pasteur and Joubert. Only in the 1930s was the discovery of sulfanilamide and penicillin ushered in with the modern era of chemotherapy of infections.

Antibiotics are chemical compounds which are totally or partly synthesized by living microorganisms that either inhibit the growth of, or kill, other microorganisms. Chemotherapy agents are compounds that are not produced by living organisms, but generally have a synthetic origin, which are used to attack and eradicate pathogenic organisms in the host without inflicting significant damage to the body [1].

Pharmacokinetic information on most other drugs has been forthcoming only in the last two or three decades, as no specific and sensitive methods were previously available. In the case of antibiotics, this type of information had emerged earlier on, thanks to the microbiological assay, which also covered pharmaceutical manufacturing before the HPLC technique came into routine use. Microbiological methods can assay the parent drug together with its active metabolite(s), namely the active moiety, which is what is required by the bioavailability definition set out in the European Union (EU) and US Food and Drug Administration (FDA) guidelines on bioavailability and bioequivalence [2–4]. However, microbiological methods employ microorganisms that are very sensitive to the given drug, whereas in the body, a number of different microorganisms can be

present that are the target to be controlled or killed by the antibiotic agent. In addition, some microorganisms can acquire resistance to given antibiotics. These factors, along with the availability of highly sensitive and specific chromatographic techniques have oriented pharmacokinetics of antibiotics towards these techniques, whereas microbiological methods are still extensively used for other research applications [5].

This review describes methods employed in pharmacokinetic applications covering β-lactam antibiotics, aminoglycosides, macrolides, tetracyclines, and the chemotherapeutic agents sulfonamides, quinolones, fluoroquinolones, chloramphenicol and thiamphenicol. Brief notes on the history, chemistry, action mechanism and pharmacokinetic behaviour are also provided, noting the original bibliographic references, where possible, in honour of the investigators who discovered these compounds.

2. β-Lactam antibiotics

2.1. History

In 1928 Alexander Fleming observed that a mould belonging to the genus *Penicillium* that was contaminating one of his staphylococcus cultures led to lysis of nearby bacteria. The broth in which the fungus was grown proved to inhibit many microorganisms to a marked degree. Fleming named this antibacterial moiety penicillin [6].

A variety of problems were solved in the next decade, to allow laboratory production that sufficed to conduct clinical trials on patients who were desperately ill with infections refractory to all other known therapeutic agents. Most of the other problems related to the industrial production of penicillin were tackled by the end of the Second World War. The cost of penicillin, when it first became available on the market, was several dollars per 100 000 units, which is about 10–20 times more than it costs today.

2.2. Chemistry and action mechanism

The basic structure of penicillin is a thiazolidine ring (I) connected to a β-lactam ring (II), to which a side chain (R) is attached (Fig. 1). Chemical or

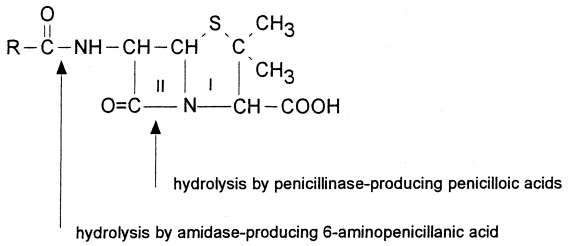


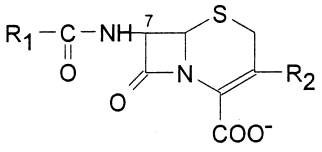
Fig. 1. Chemical structure of penicillins and bonds affected by enzymatic hydrolysis.

metabolic alterations of this nucleus lead to inactivation. The only natural penicillin now on the market is penicillin G (benzylpenicillin).

Semisynthetic penicillins are produced starting from 6-aminopenicillanic acid, obtained from cultures of *Penicillium chrysogenum*, to which side chains can be added to obtain molecules that are more resistant to β-lactamase. As bacterial resistance to β-lactam antibiotics continues to increase, efforts have been made to stabilize these molecules, with β-lactamase inhibitors like clavulanate being associated with amoxicillin and ticarcillin to extend their spectra of activity.

Penicillins are quoted in international units: one unit of crystalline sodium salt of penicillin G is 0.6 μg.

Cephalosporins possess a cephem nucleus to which two side chains are linked, one of which esterifies the carbamate group in position 7 (R_1) and the other in the nucleus (R_2) (Fig. 2). Cephalosporins are classified into four generations, according to the extent of their effect against gram-negative activity. The fourth generation includes molecules with a spectrum similar to the third, but with



R_1 = side chain that esterifies the carbamate group

R_2 = side chain in the nucleus

Fig. 2. Chemical structure of cephem nucleus.

markedly increased stability to hydrolysis by β-lactamase.

Penicillins and cephalosporins act as bacteriostatics by inhibiting bacterial peptidoglycan cell wall synthesis [7].

2.3. Pharmacokinetic profile

Some β-lactam antibiotics that are well absorbed through the gut are administered orally, while others that are poorly absorbed or have nil absorption are administered parenterally at doses ranging from 400 to 2000 mg two or three times a day. In the case of ampicillin, several prodrugs have been synthesized which generate ampicillin in the body by hydrolysis of the ester bond. These are bacampicillin, talampicillin, pivampicillin, hetacillin and lenampicillin [8]. Some of these prodrugs enhance the systemic bioavailability of ampicillin by as much as 50%.

These compounds interact to varying degrees of intensity with plasma albumin and possess a relatively low distribution volume, and, with a few exceptions (e.g. amoxicillin) [9], linear pharmacokinetics. Excretion occurs mainly through glomerular filtration, but also through tubular secretion, which can be inhibited by probenecid [10].

Most of these compounds have a short terminal half-life, $\leq$1 h. However, some second, third and fourth generation cephalosporins possess longer half-lives, e.g. cefonicid (4.4 h), ceforanide (2.6 h), cefotetan (3.6 h), ceftriaxone (8.0 h), cefoperazone (2.2 h), cefepime (2.0 h), cefetamet (2.5 h) and cefexime (3.0 h). Cefaclor is also marketed in 375-mg modified-release formulations for a b.i.d. dose turnover, which with 250-mg immediate-release formulations is t.i.d.

The main pharmacokinetic characteristics of selected cephalosporins are listed in Table 1.

2.4. Analytical methods

Various methods and reviews have been published covering the analysis of penicillins and cephalosporins in biological matrices and pharmaceutics [11–13]. These hydrophilic substances are usually analysed in the supernatant of a PCA-deproteinized plasma sample to which an internal standard is added. Autosampling injectors (50 μl/injection) are used to process analytical series, namely unknown samples, calibration curve and quality controls. UV detection usually occurs at 254–260 nm, with a few exceptions, depending on absorbance of the analyte.

Problems of stability are usually encountered with these compounds, mainly affecting plasma concentrations at room temperature. From blood sampling to plasma storage in the freezer, the following procedures should be followed to minimize loss of titre in the parent compound: test tubes containing the blood must be chilled in an ice-bath and centrifuged as soon as possible using a refrigerated ($\cong +2/+4$°C) centrifuge. The resulting plasma must then immediately be frozen in dry ice and stored in a freezer at $-20°/-80$°C.

Fig. 3 depicts typical HPLC recordings and Table 2 lists the main analytical conditions of cefaclor analysis in human plasma as well as extensions of this method to other analytes of the class.

Clavulanate, a potent, irreversible β-lactamase inhibitor, is analysed in plasma and urine through a derivatisation with benzaldehyde in the presence of phosphate buffer pH 3.8, which gives a derivative separated in a reversed-phase column that is sensitive to fluorimetric detection (λ_{ex} 386 nm, λ_{em} 460 nm) with a detection limit of 10 ng/ml [14].

3. Aminoglycosides

3.1. History

In the 1930s and 1940s, the discovery of penicillins provided a major boost to stimulate the search for other antimicrobial agents. In 1943, Schatz et al. discovered and isolated a strain of *Streptomyces griseus* to produce streptomycin, a potent antimicrobial agent that is active on *Tubercle bacillus* and various aerobic gram-positive and gram-negative microorganisms [15]. Further studies led to the development of neomycin from *Streptomyces fradiae* and kanamycin from *Streptomyces kanamyceticus* [16]. Other aminoglycosides developed around this time include amikacin, gentamicin, netilmicin and tobramycin.

3.2. Chemistry and action mechanism

Aminoglycosides consist of two or more aminosugars joined via a glycoside linkage to a hexose

Table 1
Main pharmacokinetic features of selected cephalosporins (from Refs. [64,71,77])

Drug	$t_{1/2}$ (h)	Protein interaction (%)	V_d (l/kg)	Cl plasmatic (ml/min)	Cl renal (ml/min)
First generation					
Cefadroxil	1.2	20	0.24	84	78
Cefazolin	1.8	89	0.14	67	53
Cephalexin	0.9	14	0.26	300	275
Cephalothin	0.6	71	0.26	430	225
Second generation					
Cefaclor	0.7	25	0.36	400	220
Cefamandole	0.8	74	0.16	200	190
Cefonicid	4.4	98	0.11	22	20
Ceforanide	2.6	80	0.14	40	32
Cefotetan	3.6	85	0.14	40	27
Cefoxitin	0.8	73	0.25	440	350
Cefuroxime	1.7	33	0.20	140	130
Third generation					
Cefoperazone	2.2	89	0.14	85	24
Cefotaxime	1.1	36	0.23	250	140
Ceftazidime	1.6	21	0.23	145	120
Ceftizoxime	1.8	28	0.36	150	140
Ceftriaxone	8.0	95	0.16	20	8
Fourth generation					
Cefepime	2.0	20	0.30	130	108
Cefetamet	2.5	20	0.30	140	120
Cefixime	3.0	67	0.30	90	43
Cefotiam	1.0	40	0.50	20	12
Cefpirome	1.8	10	0.21	100	74
Cefprozil	1.5	40	0.22	210	153

nucleus (e.g. streptidine or 2-deoxystreptamine) of the drug (Fig. 4).

Aminoglycosides inhibit the protein synthesis of microorganisms, resulting in a rapid, concentration-dependent bactericidal action [17]. It should be noted that other antimicrobials that affect protein synthesis are only bacteriostatic. Their postantibiotic effect allows these drugs to be administered once a day [18]. Aminoglycosides are particularly active against aerobic gram-negative bacilli and are used inter alia to treat tuberculosis, particularly in cases of suspected multiple-drug resistance.

However, these drugs can give rise to adverse reactions, including ototoxicity and nephrotoxicity, which is almost always reversible when treatment is discontinued [19].

3.3. Pharmacokinetic profile

The main pharmacokinetic characteristics of selected aminoglycosides are summarized in Table 3.

All these drugs are very hydrophilic and thus are not absorbed through the gut. When administered orally they can sterilize the intestinal contents and are excreted as such in faeces. In order to achieve a systemic effect, these drugs are administered intramuscularly or are infused intravenously, at doses ranging from 0.5 to 1.5 mg a day, given in three doses. They interact poorly with plasma proteins and possess a low distribution volume and linear pharmacokinetics. Aminoglycosides are largely excreted via urine in unchanged form [20]. Their half-lives vary to a large extent, ranging from 2–3 h with

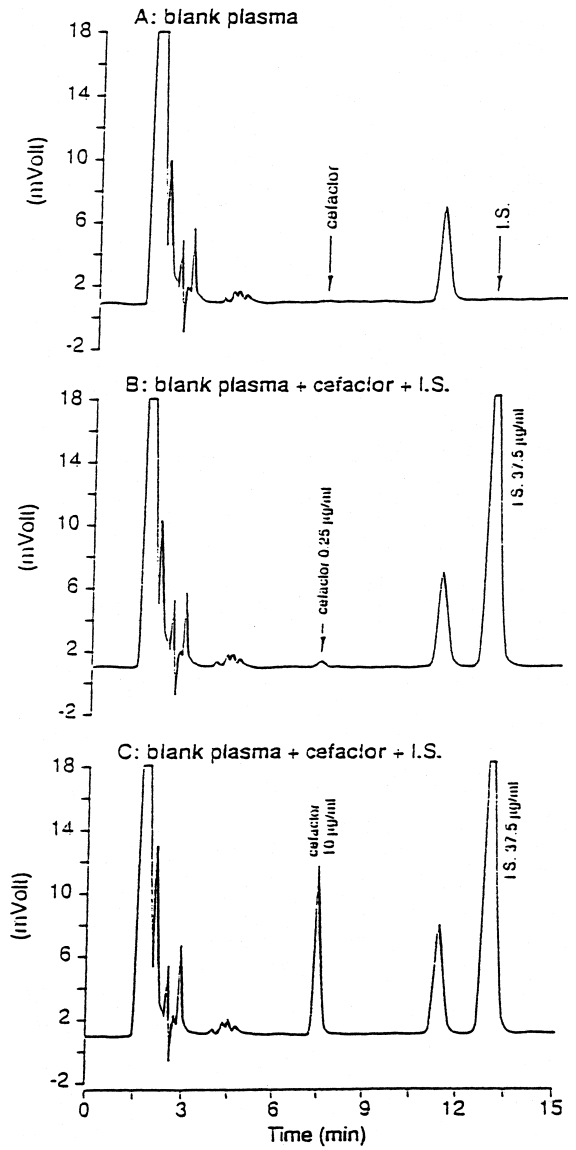


Fig. 3. Typical HPLC recordings of cefaclor assay. (A) Blank plasma; (B, C) blank plasma spiked with the analyte and internal standard (I.S.).

amikacin, kamamicin, streptomycin, through 37 h with netilmicin and up to 100 h with tobramycin.

3.4. Analytical methods

The chemical structure of aminoglycosides calls for chemical derivatisation in order to achieve the detectability needed for a HPLC assay. Fluorophore derivatives have been described in the literature [21–24]. A derivative for spectrophotometric detection was described by Papp et al. [25]. McLaughlin and Henion [26] have published a HPLC method with pulsed amperometric detection. Table 4 details analytical conditions for the assay of tobramycin in serum and urine and extensions of this method to other aminoglycosides.

4. Macrolides

4.1. History

Erythromycin, a metabolic product of a strain of *Stroptomyces erythreus*, was discovered in 1952 by McGuire and his coworkers, who described the effectiveness of this drug in experimental and naturally occurring infections by gram-positive cocci. Other compounds in this class include clarithromycin, azithromycin and roxithromycin.

4.2. Chemistry and action mechanism

The name macrolide reflects the chemical structure of this class, namely a multi-membered lactone ring to which one or more deoxy sugars are attached. Fig. 5 shows the chemical structure of erythromycin, the most important member of this class.

Macrolide antibiotics possess a bacteriostatic action that inhibits protein synthesis by binding reversibly to 50 S ribosomal subunits in sensitive microorganisms [27].

4.3. Pharmacokinetic profile

Erythromycin base is in part inactivated in the acid medium of the stomach and is incompletely absorbed through the intestine. Attempts to reduce this inactivation by administering film-coated or enteric-coated formulations, acid-stable salts (e.g. stearate, estolate) or esters (e.g. acetate, propionate, ethylsuccinate) have only been partly successful. Erythromycin and its derivatives are usually given in doses ranging from 1 to 2 g a day in 2–4 doses expressed as erythromycin base. Clarithromycin is well absorbed in the gut, but a first pass metabolism reduces

Table 2
Analytical conditions for assaying cefaclor (from Dal Bo and Marzo, unpublished data)

Analyte	Cefaclor. Cephalexin was used as an internal standard (I.S.)
Matrices	Serum, plasma, urine, 200 μl sample
Extraction procedure	200 μl of plasma and 250 μl of I.S. solution (30 μg/ml 6% PCA in water) were inserted into a 1.5-ml conical centrifuge test tube, vortex mixed and centrifuged (13 000*g*, 3 min). 50 μl of the supernatant were injected into the column
Analytical system	HPLC with UV detection at 260 nm
Column	Reversed-phase C_{18}, 250×4.6 mm I.D., 5 μm
Mobile phase	Phosphate buffer pH 2.3 containing triethylamine (1.5%, v/v) and heptanesulfonic acid (1 g/l)–methanol–tetrahydrofurane (80:16:4, v/v/v)
Flow-rate	1.4 ml/min
Retention time	Cefaclor=7.3 min I.S.=12.6 min
Quantification	Cefaclor to I.S. peak-height ratio from calibration plasma standards was used to generate a calibration curve. A weighted (1/concentration) least-square regression allowed the analyte concentration in unknown samples to be measured. Linearity was ascertained in the 0.125–30-μg/ml range Quantification limit was 0.125 μg/ml
Stability	Acceptable stability in deproteinized samples (−2.2% in 24 h at 4°C) and after two freeze/thaw cycles (−4.7%). In plasma stored at room temperature, stability dramatically decreases by 15% in 2 h
Extensions	Most β-lactam antibiotics can be assayed by HPLC after plasma clean-up carried out by deproteinization with PCA or acetonitrile [80] or by SPE [81]. Reversed-phase columns are usually employed. UV detection occurs in the 210–310-nm range, depending on analyte absorption [13]. Extraction from biological matrices can occur through a simple deproteinization and injection of supernatant, through solid-phase columns or liquid/liquid extraction [11]. Aminopenicillins and aminocephalosporins can be assayed by HPLC with fluorimetric detection of pre or postcolumn fluorescent oxidation products [82,83]. β-Lactamase inhibitor tazobactam can be assayed with HPLC methods used for most penicillins and cephalosporins [84], whereas clavulanate needs a precolumn derivatisation with imidazole for UV [85] or with benzaldehyde for fluorimetric detection [14]

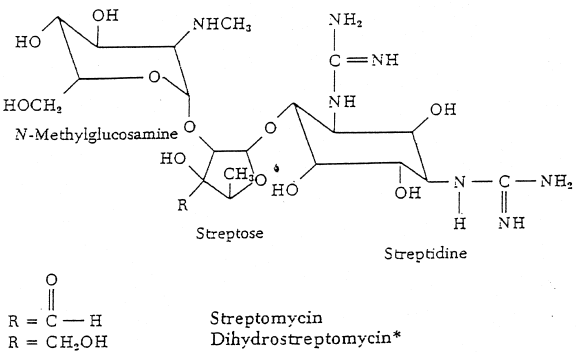


Fig. 4. Chemical structure of aminoglycosides.

its bioavailability to about 50%. It is administered at doses of 0.25–0.5 g twice a day. Azithromycin and roxithromycin are rapidly absorbed through the gut and, due to their long half-lives, can be administered once a day at a dose of 0.5–1.0 g. All these drugs diffuse readily into extracellular fluids, to produce antibacterial activity in all tissues with the sole exception of the CNS. Indeed, azithromycin possesses a distribution volume that is as high as 31 l/kg. The pharmacokinetics of azithromycin, roxithromycin and clarithromycin are dose-dependent [28].

Macrolides are mainly excreted as metabolites, with 14-hydroxyclarithromycin retaining activity [29]. Elimination half-lives are 1.6 h with erythromycin, 11 h with roxithromycin, 3–7 h with clarithromycin, 5–9 h with 14-hydroxyclarithromycin and 40 h with azithromycin. A longer half-life, namely 68 h, was found with azithromycin as an expression of distribution in a deep compartment. The main pharmacokinetic characteristics of selected macrolides are summarized in Table 5.

4.4. Analytical methods

Erythromycin has been assayed in pharmaceutics by HPLC with UV and other detectors, by GC with a flame-ionisation detector and by densitometry after TLC separation [30–32]. The microbiological, fluorimetric and spectrophotometric methods used in the 1950s and 1960s are not considered here as they are not specific. In serum, plasma and urine, erythromycin and other macrolides can be evaluated by HPLC with UV detection [33] reaching a sensitivity of 0.25 μg/ml in serum and 1 μg/ml in urine, or by HPLC with fluorimetric detection [34,35] reaching a sensitivity of 50 ng/ml. The use of electrochemical detection has emerged as the most appropriate method to analyse macrolides in pharmacokinetic and bioavailability investigations. Table 6 lists the analytical conditions for roxithromycin assay in plasma and serum using erythromycin as an internal standard. These conditions can also solve analytical problems with other macrolides and other matrices, with minor modifications. Fig. 6 shows typical HPLC recordings of roxithromycin assays.

5. Tetracyclines

5.1. History

Systematic screening led in the 1940s to the discovery and subsequent marketing of chlortetracycline, the first member of the tetracycline class. Other compounds were soon discovered to extend this class of antibiotics, which have proved to be highly effective against a number of gram-positive and gram-negative bacteria. They are thus considered

Table 3
Main pharmacokinetic features of selected aminoglycosides (from Ref. [71])

Drug	$t_{1/2}$ (h)	Protein Interaction (%)	V_d (l/kg)	Cl plasma (ml/min)	Cl renal (ml/min)
Amikacin	2.3	4	0.27	90	89
Gentamicin	53	<10	0.31	106	95
Kanamycin	2.1	nil	0.26	98	88
Netilmicin	37	<10	0.20	90	78
Streptomycin	2.6	48	0.25	84	46
Tobramycin	100	<10	0.33	70	63

Table 4
Analytical conditions for assaying selected antibiotic classes

Class	Aminoglycosides	Tetracyclines	(Fluoro)Quinolones	Rifamycins	Phenicols
Analyte(s)	Tobramycin [21]	TC, OTC, CTC [38]	Ciprofloxacin (CIPR), M_1, M_2, M_3, M_4 [55]	Rifampin (RIF), DR, FDR, FR [60]	Chloramphenicol [65]
I.S.	Gentamycin C2			Papaverine·HCl	4-(β-OH-*p*-nitrophenethyl acetamide)
Matrices	Serum, urine, 0.25 ml	Bovine and porcine muscles, 5 g	Plasma, serum, urine, bile, tissues, faeces	Human plasma, 0.5 ml	Human plasma, cerebrospinal fluid, 0.025 ml
Derivatization	With *O*-phthaldehyde		Photothermal for M_2, M_3, M_4		
Extraction	With CH_3CN and CH_2Cl_2	Buffer blending, PCA deproteinization, purification with SPE	By ultrafiltration	With $CHCl_3$	With ethylacetate and further washing with alkalinised water
Analysis system	HPLC–Fl, $\lambda_{ex}/\lambda_{em}$=340/418 nm	HPLC–UV, 360 nm	HPL–Fl, $\lambda_{ex}/\lambda_{em}$=278/445 nm	HPLC–UV, 340 nm	HPLC–UV, 254 nm
Column	μBondapak CN, 300×4 mm I.D.	Spherisorb ODS, 250×4 mm I.D., 5 μm+guard column	RP C_{18}, 250×4 mm I.D., 5 μm at 40°C	Nucleosil C_{18}, 250×4.0 mm I.D., 7 μm at 40°C	μBondapak C_{18}, 300×3.9 mm+ guard column
Mobile phase	CH_3OH 62.0 Water 35.1 CH_3CN 2.9 pH 6.5	oxalic acid 0.01 mol/l 45 CH_3CN 35 CH_3OH 20	$NBut_4HSO_4$ 40 g/l+7–10% CH_3CN for CIPR and M_1; 25–30% CH_3CN for M_2, M_3, M_4	CH_3CN 38 0.1 mol/l KH_2PO_4 62 pH 4.0	K_2HPO_4 67.5 CH_3OH 20.0 CH_3CN 12.5
Flow-rate t_R (min)	1.6 ml/min Tobramycin 8.6 I.S. 13.3	1 ml/min OTC 5.0 TC 6.3 CTC 8.8	2 ml/min M1 3.5 M3 2.5 CIPR 4.5 M4 3.5 M2 6.0	1.2 ml/min RIF 6.5 I.S. 4.3 DR 3.2 FR 14.5 FDR 5.2	2.0 ml/min Chloramphenicol 8.0 I.S. 4.0
LOQ	1 μg/ml	50 ng/g	0.23–2.2 ng/ml	0.06 μg/ml	0.25 μg/ml
Stability			One week at 4°C; >1 month at −20°C		Indefinitely at ≤−18°C
Extensions	The method is applicable to other aminoglycosides [21–24]. UV and pulsed amperometric detections were also used [25,26]	Tetracyclines can be individually assayed using another tetracycline as I.S. in tissues and plasma [39–41]. Fluorimetric [42] and electrochemical [43] detections have also been used	The method can assay other fluoroquinolones. UV detection at 340 and 275 nm was also used for other (fluoro)quinolones [56]	Using a SPE extraction and similar procedures Lau et al. [61] have assayed in human plasma rifabutin, a semisynthetic derivative of rifampin	CH_3OH or CH_3CN were also used as deproteinizing agents [66]. Thiamphenicol was detected at 224 nm [67]

Legenda: TC=tetracycline; OTC=oxytetracycline; CTC=chlorotetracycline; M_1, M_2, M_3, M_4=ciprofloxacin metabolites; DR=25-desacetyl-rifampin; FDR=3-formyl-25-desacetyl-rifamycin; FR=3-formyl-rifamycin; Fl=fluorescent detection; λ_{ex}=excitation; λ_{em}=emission.

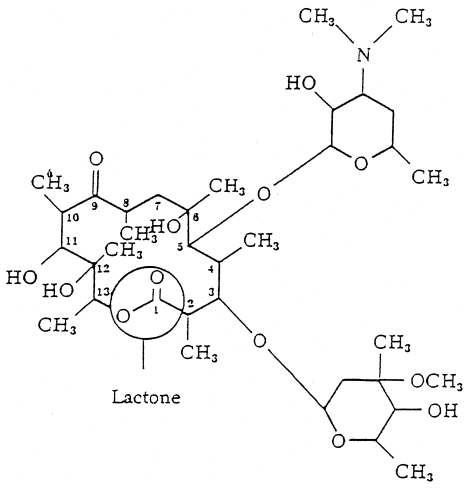


Fig. 5. Chemical structure of erythromycin.

as 'broad spectrum' antibiotics with a wide range of applications in therapeutics.

5.2. Chemistry and action mechanism

Tetrycyclines contain an octahydronaphthacene ring skeleton, consisting of four fused rings (Fig. 7) and are elaborated by *Streptomyces aurofaciens* or *Streptomyces rimosus*.

Tetracyclines inhibit the synthesis of bacterial proteins by binding to the 30 S bacterial ribosome and preventing the access of aminoacyl tRNA to the acceptor site on the mRNA–ribosome complex.

Tetracyclines are administered by the oral, parenteral and topical routes, mainly to treat diseases caused by *Rickettsiae*, *Mycoplasmas* and *Chlamydiae*. These compounds have been largely used to treat general infectious diseases and as growth additives in animal feeds, even if their veterinary use has now been abandoned in most countries, as bacterial resistance to them has dramatically increased. However, this has resulted in a number of publications reporting major improvements in analytical methods used to assay traces of these compounds in animal tissues.

5.3. Pharmacokinetic profile

Enteral absorption varies according to the individual tetracycline. In increasing order, it is low (30%) with chlortetracycline, intermediate (60–80%) with oxytetracycline and tetracycline and high with doxycycline (95%) and minocycline (100%) [20].

Tetracyclines are administered orally at doses ranging from 0.1 to 0.5 g in one or more doses a day. Enteral absorption is dose-dependent with a negative relationship. Milk, dairy products and antacids impair the enteral absorption of tetracyclines [36].

Tetrycyclines distribute widely throughout the body and into tissue secretions, including urine and the prostate gland and accumulate in the reticuloendothelial cells of various tissues, including dentine which can lead to brown discoloration of the teeth [37].

Tetracyclines are excreted mainly via urine, but also via bile into the intestine, which leads to enterohepatic circulation. Chlortetracycline, doxycycline and minocycline are partly biotransformed, and produce inactive metabolites. Elimination half-lives can vary in the 6–18 h range.

Table 7 shows the main pharmacokinetic characteristics of selected tetracyclines.

Table 5
Main pharmacokinetic features of selected macrolides (from Refs. [71–73,86])

Drug	$t_{1/2}$ (h)	Protein interaction (%)	V_d (l/kg)	Cl plasma (ml/min)	Cl renal (ml/min)
Azithromycin	40	7–50 (dose dependent)	31	630	76
Clarithromycin	3–7	42–50	2.6	511	184
Erythromycin	1.6	84	0.8	640	76
Roxithromycin	11	96	0.6	3.3	1

Table 6
Analytical conditions for assaying roxythromycin (from Dal Bo and Marzo, unpublished data)

Analyte	Roxithromycin. Erythromycin was used as an internal standard (I.S.)
Matrices	Plasma or serum, 1.0 ml
Extraction procedure	0.5 ml of Na_2CO_3 saturated solution, 1 ml of water and 4 ml of ethyl ether were added to 1 ml of plasma and 20 μl of I.S. solution (1 mg/ml in acetonitrile). The mixture was stirred in a rotating mixer and centrifuged. 3.5 ml of the upper phase were evaporated to dryness, redissolved with acetonitrile: ammonium acetate 83 mol/l (1:1), diluted with 1 ml of *n*-hexane and vortex mixed. The aqueous layer (50 μl) was injected into the column
Analytical system	HPLC with coulometric detection. Guard cell: +1 V; detector 1: +0.7 V; detector 2: +0.9 V
Column	μBondapak C_{18}, 300×3.9 mm I.D., 10 μm, protected by a guard column
Mobile-phase	Acetonitrile–methanol: 83 mol/l at pH 7.5 ammonium acetate (5:2:3, v/v/v)
Flow-rate	1 ml/min
Retention time	Roxithromycin=6.10 min I.S.=10 min
Quantification	By the peak height ratio from calibration curve covering the range from 0.25 to 20 μg/ml. Quantification limit was 0.25 μg/ml
Stability	Roxithromycin proved to be stable for 24 h in plasma at room temperature and at 4°C in the solution to be injected after three freeze/thaw cycles
Extensions	A number of HPLC methods with UV detection [74] have been published for erythromycin assay, which however lack sensitivity HPLC with fluorescent detection improves sensitivity but requires complex derivatizations [75]. HPLC with electrochemical detection has been successfully applied to trace analysis of macrolides in biological fluids [34,76]. When properly validated, the above method can be used to assay erythromycin and other macrolides, e.g. clarithromycin, 14-OH clarithromycin, in serum, plasma and also in pharmaceutics

5.4. Analytical methods

Among the tetracycline class, oxytetracycline, tetracycline, chlortetracycline and doxycycline are widely used in veterinary medicine and animal nutrition. The monitoring of these compounds and their metabolites as residues in slaughtered animals has thus become one of the most important analytical applications of tetracyclines, and it requires high sensitivity.

HPLC with UV detection has widely been employed in these assays by various authors [38–41].

Fluorimetric [42] and electrochemical detection [43] have been successfully employed. However, tetracyclines are usually assayed in plasma or serum and in tissue homogenates of various animal species by HPLC with UV detection at 350–365 nm, reaching a quantification limit as low as 10–100 ng/g or ng/ml [38,40,41,44]. A summary of these methods is given in Table 4.

6. Sulfonamides

6.1. History

Sulfonamides were the first chemotherapeutic agents discovered. In the 1930s, it was casually

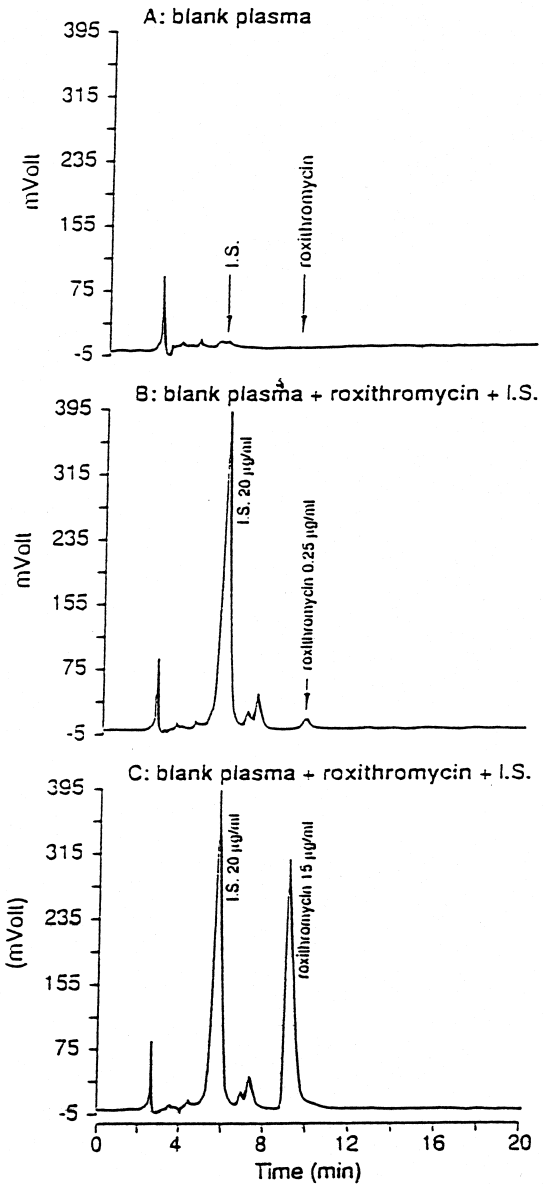
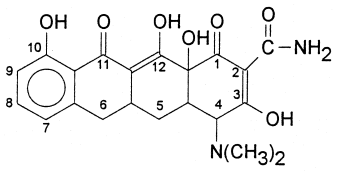


Fig. 6. Typical HPLC recordings of roxithromycin assay. (A) Blank plasma; (B, C) blank plasma spiked with the analyte and internal standard (I.S.)

noted that the azo dye prontosil rubrum, namely 2,4-diaminobenzene-4′-sulfonamide, and other similar azo dyes were active against streptococci. Domagk's discovery of the antibiotic activity of prontosil [45] resulted in his being awarded the Nobel Prize in Medicine and triggered major efforts



Substituents

Compound	Position 5	Position 6	Position 7
Chlortetracycline			-Cl
Oxytetracycline	-OH / -H		
Demeclocycline		-OH / -H	-Cl
Methacycline	-OH/-H	$=CH_2$	
Doxycycline	-OH / H	$-CH_3$/-H	
Minocycline		-H/-H	$-N(CH_3)_2$
Tetracycline		$-CH_3$/-OH	

Fig. 7. Chemical structure of tetracyclines.

in research, which led to the development of several active compounds in this class as well as compounds of other classes with relatively similar chemical structures, such as carbonic anhydrase, inhibitor-type diuretics and sulfonylurea hypoglycaemic agents.

6.2. Chemistry and action mechanism

Sulfanilamide is the basic chemical structure of various sulfonamides, which originate from various substituents in the sulfamide group (Fig. 8). Sulfonamides act as competitive antagonists of *para*-aminobenzoic acid (PABA), preventing it being used to synthesise folic acid [46]. Sensitive microorganisms must synthesize their own folic acid, whereas bacteria that can utilize preformed folate are not affected. Mammals also utilize preformed folate and are thus unaffected by this mechanism. Trimetoprim is a synergist of sulfonamides. It operates by a mechanism involving selective competitive inhibition of microbial dihydrofolate reductase, which catalyses the reduction of dihydrofolate to the active tetrahydrofolate [47].

As a first choice therapy, the use of sulfonamides has been markedly reduced by the simultaneous development of other classes of antibiotic and the gradual increase in the resistance of bacteria to the drug. However, the synergy of trimetoprim with the

Table 7
Main pharmacokinetic features of selected tetracyclines (from Refs. [64,71,77])

Drug	$t_{1/2}$ (h)	Protein interaction (%)	V_d (l/kg)	Cl plasma (ml/min)	Cl renal (ml/min)
Doxycycline	18	88	0.75	37	15
Minocycline	16	76	1.3	70	7
Tetracycline	11	65	1.5	117	68
Chlortetracycline	6	45	–	–	42
Oxytetracycline	9	20–40	1.7	150	90

sulfonamides discussed above has stimulated a revival of this kind of association.

6.3. Pharmacokinetic profile

Sulfonamides include a number of chemical entities, with specific pharmacokinetic properties. Some drugs, designed to treat infections in the bowel, are poorly or nil-absorbed in systemic circulations, whereas other compounds designed for systemic activity are well absorbed in the intestine. They usually diffuse easily in body tissue. Interaction with plasma proteins varies from drug to drug. The most significant biotransformation involves the acetylation of the aromatic amine resulting in the production of inactive metabolites, which retain the toxicity of their parent compounds. Elimination occurs mainly via urine and to a less or extent via bile and faeces. Some poorly soluble substances can precipitate causing crystalline deposits and may thus cause urinary obstruction.

The half-life of these compounds is variable, depending on various factors which include the pH of urine and renal function.

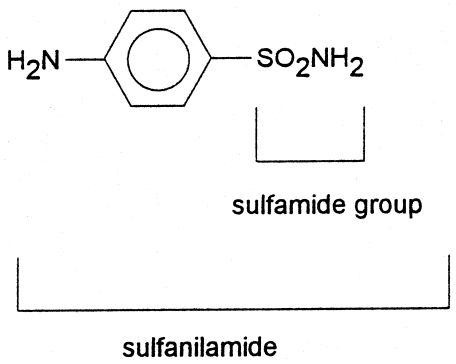


Fig. 8. Chemical structure of sulfanilamide. Sulfonamides derive from the substitution in the sulfamide group.

6.4. Analytical methods

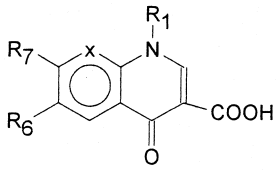
The analytical methods to assay sulfonamides have been comprehensively reviewed by Horowitz [48] and further by Agarwal [49]. These compounds are usually extracted from the matrix into organic solvents or are purified by SPE and analysed by HPLC. Assay can occur by UV detection in the range 265–310 nm, by fluorometric detection after chemical derivatisation with dimethylaminobenzaldehyde, or by electrochemical detection.

As associations between sulfonamides and trimetoprim and its congeners are of wide therapeutic use, some methods can assay both these active ingredients. Ascalone [50] used a normal-phase liquid chromatographic method, whereas other authors have used reversed-phase conditions [51].

7. Quinolones

7.1. History

The first member of this class, nalidixic acid, was discovered casually in 1962 by Lescher and his coworkers, and appeared to be of interest in treating infections of the urinary tract, due to its action against aerobic gram-negative microorganisms. However, nalidixic acid and a series of further compounds in the class, eventually proved to be of relatively minor importance due to their limited therapeutic effect, poor systemic concentrations and rapid development of bacterial resistance. Renewed interest was devoted in the 1980s to developing



where:

x = N in nalidixic acid, -CH- or -CF- in other compounds

R_1 and R_7 are different substituents

R_6 is F in fluoroquinolones

Fig. 9. Chemical structure of quinolones and 6-fluoroquinolones.

fluoroquinolones, which are active by the oral route, have higher distribution volumes and broader antimicrobial activity and are very well tolerated. These compounds are now used therapeutically to treat a wide variety of infectious diseases including urinary tract infections.

7.2. Chemistry and action mechanism

The chemical structures of quinolones and fluoroquinolones is depicted in Fig. 9. Fluoroquinolones have a fluorine in position 6 and many other compounds contain a piperezine ring in position 7.

Quinolones act by inhibiting gyrase-mediated DNA supercoiling at concentrations overlapping with those required to inhibit bacterial growth (0.1–10 μg/ml). The bacterial enzyme DNA gyrase controls the introduction of negative supercoils into DNA. The first members of this class were used only to treat urinary tract infections. The most recently developed fluoroquinolones are also effective in systemic therapy, such as prostatitis, infections of the gastrointestinal tract, respiratory tract, bone, joint and soft tissues and sexually transmitted diseases.

7.3. Pharmacokinetic profile

Quinolones are well absorbed through the gut and fluoroquinolones are widely distributed. However distribution volume is low with quinolones (0.33 l/kg with cinoxacin). This increases to 1.8 l/kg with ciprofoxacin and ofloxacin, 1.5–2.0 l/kg with pefloxacin, 2.3 l/kg with lomefloxacin, and 3.2 l/kg with norfloxacin (Table 8). The half-lives of fluoroquinolones are also higher than those of quinolones, reaching 5.0 h with norfloxacin, 8.0 h with lomefloxacin and 6–14 h with pefloxacin. Norfloxacin is the active metabolite of pefloxacin. Pefloxacin proved to possess linear kinetics after both i.v. and oral administration of 200, 400, 600 and 800 mg [52]. In comparison with quinolones, their higher distribution volumes, longer half-lives and good tolerability allow some fluoroquinolones to be administered once-a-day in repeated dose regimens. Most quinolones and fluoroquinolones are administered orally at doses ranging between 0.2 and 0.5 g every 12 h.

7.4. Analytical methods

Various analytical methods for the assay of quinolones and fluoroquinolones are described.

Clean up from the matrix can be carried out by:

Table 8
Main pharmacokinetic features of selected quinolones and fluoroquinolones (from Refs. [71,78,79])

Drug	$t_{1/2}$ (h)	Protein interaction (%)	V_d (l/kg)	Cl plasma (ml/min)	Cl renal (ml/min)
Cinoxacin	2.1	63	0.33	175	125
Ciprofloxacin	4.1	40	1.8	420	270
Lomefloxacin	8.0	10	2.3	230	150
Nalidixic acid	2–3	80	–	–	6.5
Norfloxacin[a]	5.0	20	3.2	500	150
Ofloxacin	5.7	25	1.8	245	156
Pefloxacin	6–14	20–30	1.5–2.0	100–160	7–22

[a] Norfloxacin is the active metabolite of pefloxacin.

- liquid–liquid extraction with CH_2Cl_2, $CHCl_3$, or $CHCl_3$–isopentanol (9:1, v/v) [53]
- SPE [54]
- PCA denaturation or ultrafiltration [55]

Detection can be managed in fluorescence, taking advantage of the spontaneous fluorescence of most compounds in this class [56]. Fluorescence can be induced or enhanced by post-column photothermal reaction. Spectrophotometric detection can also be used, but it is generally less sensitive.

Table 4 lists analytical conditions used to assay ciprofloxacin in biological matrices as well as extensions to other fluoroquinolones.

8. Other compounds

8.1. Rifamycins

8.1.1. General description of the class

The rifamycin group comprises structurally similar complex macrocyclic antibiotics, produced by *Streptomyces mediterranei*. Rifampin (Fig. 10), the most common compound in the class, inhibits the growth of most gram-positive and some gram-negative microorganisms by acting on DNA-dependent RNA polymerase and forming a stable drug–enzyme complex which results from suppressing the initiation of chain formation in RNA synthesis [57]. Rifampin on its own or in association with isoniazid is employed in treating tuberculosis [58].

Rifampin is well absorbed by the gut, is widely distributed in body tissues (the distribution volume is about 1 l/kg) and is excreted mainly via the bile, activating an enterohepatic circulation [59]. The metabolic rate of rifampin increases as the treatment is prolonged as a result of the induction of liver microsomal enzymes involved in its deacetylation.

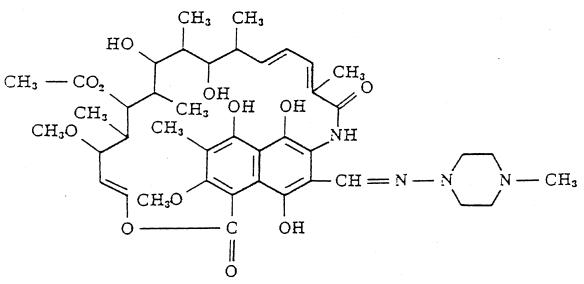


Fig. 10. Chemical structure of rifampin.

8.1.2. Analytical methods

Ishi and Ogata [60] have assayed rifampin, called also rifampicin, and its metabolites 25-desacetyl-rifampin, 3-formyl-25-desacetyl-rifamycin SV and 3-formyl-rifamycin SV in human plasma by HPLC with a solvent extraction and HPLC with UV detection. Using similar procedures, Lau et al. [61] have assayed the semisynthetic derivative of rifampin, rifabutin, in human plasma after a SPE filtration and HPLC with UV detection. A summary of these methods is given in Table 4.

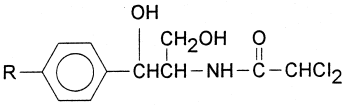
8.2. Chloramphenicol and thiamphenicol

8.2.1. General description

Chloramphenicol is an antibiotic produced by *Streptomyces venezuelae*, which was first employed in the late 1940s to treat a typhus epidemic in Bolivia [62]. Since 1950, the use of this drug has been confined to patients suffering from very serious infections who could not take safer alternatives because of resistance or allergies. In the late 1950s, thiamphenicol was discovered by Cutler et al. [63], who succeeded in changing the $-NO_2$ group of chloramphenicol for the CH_3SO_2- group of thiamphenicol (Fig. 11). The resulting drug proved to be safer, being devoid of the typical blood toxicity of chloramphenicol.

These substances inhibit protein synthesis in bacteria and, to a lesser extent, in eukaryotic cells, by binding reversibly to the 50 S ribosomal subunit.

Both chloramphenicol and thiamphenicol are well absorbed through the gut and diffuse in the body



where:

R = NO_2- in chloramphenicol

R = CH_3SO_2- in thiamphenicol

Fig. 11. Chemical structure of chloramphenicol and thiamphenicol.

tissues. Interaction with plasma proteins is about 50% with chloramphenicol and 20–25% with thiamphenicol. Both the drugs are excreted via urine, chloramphenicol mainly as a glucuronide, and thiamphenicol mainly as such [64].

8.2.2. Analytical methods

Chloramphenicol was assayed in biological matrices (plasma, serum, cerebrospinal fluid, etc.) after solvent extraction [65] or deproteinisation with methyl alcohol or acetonitrile [66] and was quantified with UV detection using an internal standard. Similarly, thiamphenicol was assayed in biological matrices [67].

Here again these methods have also been adopted to assay these compounds in the tissues of food-producing animals. In this case, sensitivity was improved starting from a greater amount of matrix and using a multi-step clean up procedure [68].

A synoptic view of these methods is presented in Table 4.

9. Discussion

In the late 1970s and 1980s, the use of HPLC spread widely and solved various analytical problems, including applications in pharmacokinetics. The need for specificity in these assays compelled operators of this area to shift from microbiological to HPLC assays. Most analytical problems benefited from UV detection without derivatisation. However, some classes of antibiotic required a chemical derivatisation in order to render the analyte sensitive to UV or fluorometric detection. This is the case with aminoglycosides and macrolides, which however can also be detected electrochemically without derivatisation. These chromatographic techniques are usually sensitive enough for most antibiotics as they achieve a limit of quantification (LOQ) of 0.30–0.5 μg/ml.

However increased sensitivity is required for specific applications, such as assaying these compounds in the saliva [69], or to monitor the plasma concentration of some compounds which are segregated in a deep compartment and thus possess a markedly high terminal half-life (e.g. tobramycin which has a $t_{1/2}$ of 100 h). Plasma concentrations in bioavailability and bioequivalence studies must be monitored over a period enough to extrapolate AUC to infinity by adding ≤20% of the total extrapolated AUC. This means that plasma concentrations must be monitored up for ≥3 fold their half-lives, even if they are fully inactive concentrations [4]. As various antibiotics are used in veterinary practice, operating procedures require these substances to be detected in meat to be sold. The high sensitivity required for these specific applications can be achieved with HPLC coupled with fluorometric or with MS detection or with the MS–MS technique.

The high specificity of HPLC allows the parent drug and its metabolites to be assayed simultaneously, mainly when they retain activity and thus are included in the active moiety. Examples of antibiotics which produce active metabolites in vivo are pefloxacin, cefotaxime and clarithromycin. These are biotransformed into norfloxacin, desacetyl-cefotaxime and 14-OH-clarithromycin, respectively [70].

Some antibiotics, e.g. β-lactams, have serious stability problems, which call for carefully defined procedures in analytical manipulation and matrix storage.

The authors conclude that the HPLC technique, using various kinds of detectors, namely UV, fluorometric, electrochemical and mass spectrometry, with or without chemical derivatisations, possess the needed sensitivity and specificity to assay all antibiotics reviewed here for pharmaceutical, pharmacokinetic, metabolic and bioavailability purposes.

10. Abbreviations

AUC	area under the plasma concentration–time curve
b.i.d.	bis in die
Cl	clearance
CNS	central nervous system
FL	fluorescence detection
HPLC	high-performance liquid chromatography
I.S.	internal standard
LOQ	limit of quantification
PABA	*para*-aminobenzoic acid
PCA	perchloric acid
SPE	solid-phase extraction
t.i.d.	ter in die
$t_{1/2}$	half-life
UV	ultraviolet
V_d	distribution volume

References

[1] O. Le Roy Salerni, Natural and Synthetic Organic Medicinal Compounds, C.V. Mosby, St. Louis, MO, 1976, pp. 166–224.
[2] EU note for guidance: Investigation of bioavailability and bioequivalence. CPMP working party on efficacy of medicinal product. III/54/89-EN-FINAL, December 1991.
[3] US Pharmacopoeia XXIII Suppl. 5 (1996) 3490.
[4] A. Marzo, L.P. Balant, Arzneim. Forsch. 45 (1995) 109.
[5] F. Jehl, C. Gallion, H. Monteil, J. Chromatogr. 531 (1990) 509.
[6] A. Fleming (Ed.), Penicillin: Its Practical Application. Blakiston, Philadelphia, PA, 1946, pp. 1–33.
[7] J.T. Park, J.L. Strominger, Science 125 (1957) 99.
[8] A. Marzo, N. Monti, M. Ripamonti, E. Arrigoni Martelli, M. Picari, J. Chromatogr. 507 (1990) 235.
[9] D.A. Spyker, R.J. Rugloski, R.L. Vann, W.M. O'Brien, Antimicrob. Agents Chemother. 11 (1977) 132.
[10] L. Balant, P. Dayer, R. Auckenthaler, Clin. Pharmacokinet. 10 (1985) 101.
[11] A.M. Brisson, J.B. Fourtillan, J. Chromatogr. 223 (1981) 393.
[12] I. Wouters, S. Hendrickx, E. Roets, J. Hoogmartens, H. Vauderhaeghe, J. Chromatogr. 291 (1984) 59.
[13] J.O. Miners, J. Liq. Chromatogr. 8 (1985) 2827.
[14] J. Haginaka, H. Yasuda, T. Uno, J. Chromatogr. 377 (1986) 269.
[15] A. Schatz, S. Bugie, S.A. Waksman, Proc. Soc. Exp. Biol. Med. 57 (1944) 244.
[16] S.A. Waksman (Ed.), Streptomycin: Nature and Practical Applications, The Williams and Wilkins Co., Baltimore, 1949.
[17] L.E. Bryan, H.M. Van Den Elzen, Antimicrob. Agents Chemother. 12 (1977) 163.
[18] M.L. Barclay, E.J. Begg, K.G. Hickling, Clin. Pharmacokinet. 27 (1994) 32.
[19] W.M. Bennet, Clin. Exp. Pharmacol. Physiol. 16 (1989) 1.
[20] R.R. Bailey, Drugs 22 (1981) 321.
[21] D.B. Haughey, D.M. Janicke, M. Adelman, J.J. Schentag, Antimicrob. Agents Chemother. 17 (1980) 649.
[22] J.P. Anhalt, S.D. Brown, Clin. Chem. 24 (1978) 1940.
[23] D.A. Stead, R.M.E. Richards, J. Chromatogr. B 675 (1996) 295.
[24] G.C. Gerhardt, C.D. Salisbury, J.D. MacNeil, J. Assoc. Off. Anal. Chem. 77 (1994) 765.
[25] E.A. Papp, C.A. Knupp, R.H. Barbhaiya, J. Chromatogr. 574 (1992) 93.
[26] L.G. McLaughlin, J.D. Henion, J. Chromatogr. 591 (1992) 195.
[27] A. Brisson-Noël, P. Trieu-Cuot, P. Courvalis, J. Antimicrob. Chemother. 22(Suppl. B) (1988) 13.
[28] S.Y. Chu, L.T. Sennello, S.T. Bunnell, L.L. Varga, D.S. Wilson, R.C. Sonders, Antimicrob. Agents Chemother. 36 (1992) 2447.
[29] F. Fraschini, F. Scaglione, G. Demartini, Clin. Pharmacokinet. 25 (1993) 189.
[30] L. Elrod Jr., R.D. Cox, A.C. Plasz, J. Pharm. Sci. 71 (1982) 161.
[31] K. Tsuji, J.H. Robertson, Anal. Chem. 43 (1971) 818.
[32] C. Radecka, W.L. Wilson, D.W. Hughes, J. Pharm. Sci. 61 (1972) 431.
[33] C. Stubbs, J.M. Haigh, I. Kanfer, J. Pharm. Sci. 74 (1985) 1126.
[34] G.S. Duthy, J. Liq. Chromatogr. 7 (1984) 1023.
[35] O.G. Nilsen, T. Aamo, K. Zahlsen, P. Svarva, Diagn. Macrobiol. Infect. Dis. 15 (1992) 71S.
[36] P.J. Neuvonen, G. Gothini, R. Hackman, K. Björksten, Br. Med. J. IV (1970) 532.
[37] E.R. Grossman, A. Walcheck, H. Freedman, Pediatrics 47 (1971) 567.
[38] J. Sokol, E. Matisova, J. Chromatogr. A 699 (1994) 75.
[39] M.D.F. Santos, H. Vermeersch, J.P. Remon, M. Schelkens, P. De Backer, R. Ducatelle, F. Haesebrouck, J. Chromatogr. B 682 (1996) 301.
[40] J.R. Walsh, L.V. Walker, J.J. Webber, J. Chromatogr. 596 (1992) 211.
[41] H. Oka, H. Matsumoto, K. Uno, K.I. Harada, S. Kadowaki, M. Suzuki, J. Chromatogr. 325 (1985) 265.
[42] K. Iwaki, N. Okumura, M. Yamazaki, J. Chromatogr. 623 (1992) 153.
[43] W. How, E. Wang, Analyst (London) 114 (1989) 699.
[44] W.A. Moats, J. Chromatogr. 358 (1986) 253.
[45] G. Domagk, Dtsch. Med. Wochenschr. 61 (1935) 250.
[46] D.D. Woods, Br. J. Exp. Pathol. 21 (1940) 74.
[47] G.H. Hitchings, N.Y. Ann. Acad. Sci. 23 (1961) 700.
[48] W. Horowitz, J. Assoc. Off. Anal. Chem. 64 (1981) 104.
[49] V.K. Agarwal, J. Chromatogr. 624 (1992) 411.
[50] V. Ascalone, J. Chromatogr. 224 (1981) 59.
[51] A. Weber, K.E. Opheim, G.R. Siber, J.F. Ericson, A.L. Smith, J. Chromatogr. 278 (1983) 337.
[52] J. Barre, G. Houin, J.P. Tillement, J. Pharm. Sci. 73 (1984) 1379.
[53] G. Montay, J.P. Tassel, J. Chromatogr. 339 (1985) 214.
[54] R. Teng, T.G. Tensfeldt, T.E. Liston, G. Foulds, J. Chromatogr. B 675 (1996) 53.
[55] H. School, K. Schmidt, B. Weber, J. Chromatogr. 416 (1987) 321.
[56] C.Y. Chan, A.W. Lam, G.L. French, J. Antimicrob. Chemother. 23 (1989) 597.
[57] E. Atlas, M. Turck, Am. J. Med. Sci. 256 (1968) 247.
[58] J.W. Raleigh, Am. Rev. Resp. Dis. 105 (1972) 397.
[59] S. Furesz, R. Scotti, R. Pallanza, E. Mapelli, Arzneim. Forsch. 17 (1967) 534.
[60] M. Ishi, H. Ogata, J. Chromatogr. 426 (1988) 412.
[61] Y.Y. Lau, G.D. Hanson, B.J. Carel, J. Chromatogr. B 676 (1996) 125.
[62] J. Ehrlich, D. Gottlieb, P.R. Burkholder, L.E. Anderson, T.G. Prindham, J. Bacteriol. 56 (1948) 467.
[63] R.A. Cutler, R.J. Stenger, C.M. Suter, J. Am. Chem. Soc. 74 (1952) 5475.
[64] F. Fraschini, A. Nicolin, Manuale di Chemioterapia, Casa Editrice Ambrosiana, 3rd ed., Milan, 1995.
[65] S.P. Sood, V.I. Green, C.L. Bailey, Ther. Drug Monitor. 9 (1987) 347.
[66] S.H. Petersdorf, V.A. Raisys, K.E. Opheim, Clin. Chem. 25 (1979) 1300.

[67] L. Felice, E.H. Abdennebi, M. Ashraf, J. Assoc. Off. Anal. Chem. 71 (1988) 1156.
[68] T. Nagata, M. Saeki, J. Chromatogr. 565 (1991) 471.
[69] V. Ascalone, L. Dal Bo, J. Chromatogr. 273 (1983) 357.
[70] A. Marzo, F. Scaglione, in: F. Fraschini, A. Nicolin (Eds.), Manuale di chemioterapia, Casa Editrice Ambrosiana, 1995, pp. 83–96.
[71] Goodman and Gilman, in: J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon, A. Goodman Gilman (Eds.), The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, 9th ed., 1996, pp. 1712–1792.
[72] N.J. Lalek, D.L. Morris, Clin. Pharmacokinet. 25 (1993) 370.
[73] S.K. Puri, H.B. Lassman, J. Antimicrob. Chemother. 20(Suppl. B) (1987) 89.
[74] K. Tsuji, M.P. Kane, J. Pharm. Sci. 71 (1982) 1160.
[75] K. Tsuji, J. Chromatogr. 158 (1978) 337.
[76] S. Laasko, M. Scheinin, M. Anttila, J. Chromatogr. 526 (1990) 475.
[77] Martindale: The Extra Pharmacopoeia, Pharmaceutical Press, London, 30th ed., 1993, pp. 79–224.
[78] G. Cuisnaud, N. Ferry, N. Pozet, P.Y. Zech, J. Sassard, Br. J. Clin. Pharmacol. 14 (1982) 489.
[79] F. Bressolle, F. Gonçalves, A. Gouby, M. Galtier, Clin. Pharmacokinet. 27 (1994) 418.
[80] K. Lindgren, J. Chromatogr. 413 (1987) 351.
[81] W.J.J. Krauwinkel, N.J. Volkers-Kamermans, J. Van Zijtveld, J. Chromatogr. 617 (1993) 334.
[82] K. Miyazaki, K. Ohtani, K. Sunada, T. Arita, J. Chromatogr. 276 (1983) 478.
[83] H. Mascher, C. Kikuta, J. Chromatogr. 506 (1990) 417.
[84] A.P. Okampo, K.D. Hoyt, N. Wadgaonkar, A.H. Carver, C.V. Puglisi, J. Chromatogr. 496 (1989) 167.
[85] I.D. Watson, J. Chromatogr. 337 (1985) 301.
[86] O.G. Nilsen, J. Antimicrob. Chemother. 20(Suppl B) (1987) 81.